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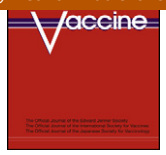

Epstein–Barr virus genome load is increased by therapeutic vaccination in HIV-l carriers, and further enhanced in patients with a history of symptomatic primary infection

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ABSTRACT

Objective: Epstein–Barr virus (EBV) infection is an established risk factor for B-cell lymphomas in Human Immunodeficiency virus (HIV)-1 infected patients. A disturbed EBV-host relationship is seen in patient groups with a high risk for EBV-associated lymphomas. We have analysed this relationship by measuring EBV-DNA in the blood of HIV-1 carriers.
Method: EBV-DNA load in B-cells was monitored by PCR in non- or insufficiently antiretroviral treated and rgp160-vaccinated HIV-patients.
Results: Both asymptomatic HIV-infected and AIDS-patients showed a 25–40-fold increase in the number of B cell associated EBV-DNA copies compared to healthy controls. Patients included in a vaccine trial with recombinant HIV gp160 showed a 5-fold increase of EBV load compared to non-immunised patients and a 50-fold increase compared to healthy controls. There was no difference whether they received vaccine or "placebo". Vaccinated patients with a history of symptomatic primary HIV-1 infection (PHI) had a 280-fold increase in median EBV load compared to healthy controls, thus suggesting a synergistic effect between the vaccination and PHI, which hypothetically could affect lymphoma risk.
Conclusions: We recommend analysis of EBV-load and long term follow up of lymphoma risk in all therapeutic HIV-1 vaccination trials.

1. Introduction

Epstein–Barr virus (EBV) is present in more than 90% of all human adults and establishes lifelong latency in B cells in the human host after primary infection [1]. When immune control is suppressed the virus can be reactivated as for example in transplanted individuals [2]. Latent EBV infection in B lymphocytes is likely to be a risk factor for B-cell lymphomas in conditions of combined antigen stimulation and immunosuppression, e.g. in holoendemic malaria, after transplantation, and in human immunodeficiency virus (HIV)-1 induced immunodeficiency [3].

Before the introduction of anti-retroviral therapy, the risk of developing B-cell lymphomas in HIV-1 seropositive patients was several thousand fold higher than in HIV-1 sero-negative persons of the same age group [4]. Thirty–forty percent of the peripheral lymphomas and close to 100% of the primary central nervous system (CNS) lymphomas were EBV-positive [5]. In post-mortem examinations, 19% of AIDS-patients were diagnosed to have non Hodgkin lymphoma [6]. The lymphoma risk, especially the primary CNS lymphomas, has significantly decreased, in the era of combination anti-retroviral therapy (cART) [7]. An over-risk for non-Hodgkin lymphomas (NHL) still remains in HIV-infected patients [8,9].

Defective T-cell immunity in patients has previously been shown to result in an abnormally high number of EBV-infected B cells in blood, e.g. in chronic active EBV infection, post-transplant patients and in HIV-infected patients [10,11].

Vaccination including adjuvant may affect the EBV-host balance, especially in immunocompromised individuals, e.g. those

Abbreviations: EBV, Epstein–Barr virus; HIV, Human Immunodeficiency virus; PHI, history of symptomatic primary HIV-infection.

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with HIV-1 infection as it affects HIV-1-, EBV-, CMV- and/or HCV-specific CD4 T-cells [12,13]. This vaccination effect on specific CD4 T-cells might in turn also affect the B-cell compartment [14,15]. A history of symptomatic primary HIV-1 infection (PHI) is also known to affect the composition of the B-lymphocyte pool [16].

In this paper we analyse subgroups of non- or insufficiently antiretroviral treated HIV-infected patients and their EBV-host relation measured by EBV genome load. Vaccination with recombinant HIV-1 gp160 (rgp160)/adjuvant and symptomatic primary HIV-infection (PHI) both affects B-cell function. We show an increased EBV-load in blood B-cells after therapeutic vaccination and a further enhancement of EBV-DNA in patients with a history of PHI.

2. Material and methods

2.1. Patients and controls

Sixty HIV-1 positive patients from the outpatient clinic at Huddinge hospital and/or South Hospital, Stockholm, including 42 participants in vaccine trials were randomly selected for this study (Tables 1 and 2). After informed consent 20 mL of blood was collected. HIV-1 negative controls (not matched for age, sex or risk group) were selected among healthy laboratory personnel. Permission for the study was obtained from the regional Ethical Committee at the Karolinska Institute (#51/97). Of the 42 immunised individuals, 32 participated for two years in a double blind placebo controlled phase III vaccine trial with r160 (rgp160)/"placebo" [17]. Both in the rgp160 vaccine and placebo arm alum was included as adjuvant. In this early vaccine trial patients received at least eight vaccinations with alum/rgp160 at regular intervals for 21 months. Placebo was given according to the same time schedules. The other 10 patients were during more than three years included in an on-going open phase II clinical study with the same vaccine. These patients got 12–16 vaccinations during three years.

The patients were treated during the pre-HAART/cART era with one or two nucleoside analogues or foscarnet. We designate this treatment regimen as insufficient antiretroviral therapy, as indicated both by CD4 counts and breakthroughs of HIV RNA levels.

Sex, age, patient origin, route of transmission, CD4+ and CD8+ cell counts are summarised in Table 1.

Throat washes for EBV DNA analysis were collected at the same time as blood samples from 31 of the HIV-1 seropositive patients included in the rgp160 vaccine trials and from three HIV-1 seropositive patients who were not included in any of the rgp160 vaccine trials. The patients were asked to gargle for 30 s with 20 ml of 0.9% sodium chloride.

2.2. EBV serology

EBV IgG antibody titers to EA and VCA was determined in plasma by conventional immunofluoroscence applied to antigen positive cells. IgG and IgM titers were determined against EBNA 1 with peptide (p107) based ELISA.

2.2.1. DNA preparation from saliva

The patients gargled with 10 mL of RPMI medium for 1 min. The throat wash was centrifuged at 2000 rpm (approximately $600 \times g$) for 10 min, and then the supernatant was frozen at $-70\,^{\circ}C$ until testing. Half mL of the sample was lysed in 0.5 mL of PCR-lysate buffer [18].

2.3. EBV genome load

EBV DNA analysis and statistics were performed as previously reported by Friis et al. [18]. This method is as sensitive and gives similar results as quantitative PCR (qPCR) [2]. In addition it provides results in all samples, while qPCR may fail more often due to inhibition and quenching.

One hundred μL of plasma were lysed in 100 μL PCR-lysate buffer. Plasma samples were tested for positive respectively negative reaction using the same PCR condition as for blood.

2.3.1. Statistical considerations

Non-parametric Mann Whitney or Kruskal Wallis tests were applied, using StatView II (Abacus Concepts Inc.). Multivariate analysis was also performed using Simca-P 8.0 (Umetrics AB) but did not add anything to our interpretation based on univariate analysis.

3. Results

HIV-1 infected patients included in the rgp160 vaccine trials showed higher median EBV-DNA load, 2.4 copies per 1000 B cells ($n = 42$) compared to non-vaccinated HIV-carriers, 0.49 per 1000 B cells ($n = 18$; $p < 0.01$, Fig. 1A). Although the patients were recruited from two slightly different vaccination trials (see Materials and Methods), we found no statistical difference in EBV-DNA load between the two groups. A considerable individual variation was observed. There was no significant statistical difference as regards age, sex, and antiretroviral treatment when comparing immunised and non-immunised patients (Table 1). However, in the rgp160 study group higher CD4+ cell counts were detected, which is most likely a result of the selection criteria for the vaccine trial. The immunised group had a median value of 270×10^6 cells/L ($n = 42$)

Table 1
Summary of patient characteristics.

Sex (M/F)[a]	Median age (range)	Geographic origin[a] (Eur/non-Eur)	Route of transmission (HS/IV/Het)[a]	Median CD4 (range)	Median CD8 (range)
HIV-1 asymptomatic					
7/1	38	7/1	6/2/0	370 (120–810)	1200 (570–3840)
HIV-1 symptomatic					
10/0	42	8/2	9/1/0	25 (3–200)	480 (70–900)
HIV-1 vaccinated asymptomatic[b]					
32/5	37	30/5	24/2/11	290 (7–580)	930 (250–2590)
HIV-1 vaccinated symptomatic					
5/0	43	5/0	5/0/0	70 (10–360)	680 (340–930)
HIV-1 seronegative					
5/5	30	9/1	–	NT	NT

NT = not tested.
[a] M: male; F: female; Eur: European; non-Eur: non-European; HS: homosexual; IV: Intravenous drug abuse, Het: heterosexual
[b] Included in vaccine trial receiving rgp160 or placebo, alum.

Table 2
Patient data.

No	EBV genome load[a]	CD4[b]	CD8[b]	Active vaccine or Alum[c]	Antiviral treatment	Documented primary infection	History >10 years of HIV infection	Other
HIV asymptomatic								
1	0.13	230	1890	no	AZT			
2	0.19	340	1540	no	ACV[d](2 days)			
3	0.89	330	570	no	-		+	
4	0.19	510	1700	no	–	+		
5	0.031	120	880	no	–			
6	0.29	610	770	no	AZT, DDI		+	
7	2.0	390	830	no	–			
8	0.43	810	3840	no	–			
HIV symptomatic								
1	1.2	30	520	no	AZT			
2	2.4	200	760	no	AZT			HLP[g]
3	0.027	3	150	no	–			
4	0.49	4	70	no	–			HLP
5	0.53	20	580	no	–			
6	6.7	30	350	no	AZT			HLP
7	2.7	10	440	no	–			HLP
8	0.75	9	640	no	ACV, DDI			
9	2.0	40	410	no	AZT, DDI			
10	0.17	110	900	no	–			
HIV vaccinated asymptomatic[e]								
1	0.36	270	1050	A	Foscavir			
2	53	170	1510	vaccine	–			Lymphoma
3	3.3	360	1900	A	–	+		
4	0.99	240	1070	vaccine	AZT			
5	0.29	360	910	vaccine	–			
6	400	320	2590	vaccine	–	+		
7	4.6	120	510	vaccine	ACV			
8	8,8	270	1100	A	ACV		+	
9	11	210	650	A	DDC, ACV		+	
10	4.0	220	1190	A	–		+	
11	63	290	1080	A	–			
12	0.080	270	1120	vaccine	–	+		
13	0.090	200	1100	vaccine	–	+		
14	2.1	170	640	A	AZT	+		
15	5.3	480	1030	vaccine	–	+		
16	0.11	430	1680	A	–		+	
17	0.43	440	1900	vaccine	–		+	
18	2.2	180	1460	vaccine	AZT, DDI			
19	0.043	230	870	A	–			
20	2.4	100	380	vaccine	–	+		
21	1.2	240	580	vaccine	–			
22	0.42	510	1380	A	–			
23	1.2	360	800	vaccine	–			
24	3.2	560	800	A	–			
25	7.8	580	340	A	–			
26	23	490	790	A	–	+		
27	5.6	410	730	A	–			
28	0.25	370	610	vaccine[f]	–			
29	3.6	190	670	vaccine[f]	–			
30	4.2	470	500	vaccine[f]	–			
31	90	310	1050	vaccine[f]	–	+		
32	2.4	90	290	vaccine[f]	AZT, DDI			
33	0.49	370	1230	vaccine[f]	–		+	
34	0.04	7	250	vaccine[f]	AZT			
35	34	160	1540	vaccine[f]	AZT, DDI			
36	2.0	440	930	vaccine[f]	–			
37	3.0	340	810	vaccine[f]	–			
HIV vaccinated symptomatic								
1	2.3	360	680	vaccine	–			HLP
2	80	110	520	A	DDI	+		Kaposis sarcoma, epithelial cancer
3	0.17	70	740	A	Foscavir			
4	2.0	10	340	vaccine	AZT			HLP
5	1.2	40	930	vaccine	–			Kaposis sarcoma, HLP

+ = history of symptomatic primary infection (PHI), – = no antiviral drug,
[a] Number of EBV genomes per 10^3 B-cells.
[b] Number of 10^6 cells per liter of blood.
[c] Recombinant active vaccine (vaccine), Alum (A), or no vaccine (no).
[d] Acyclovir.
[e] Includes placebo.
[f] Phase II, open rgp160 study.
[g] Hairy lekoplakia.

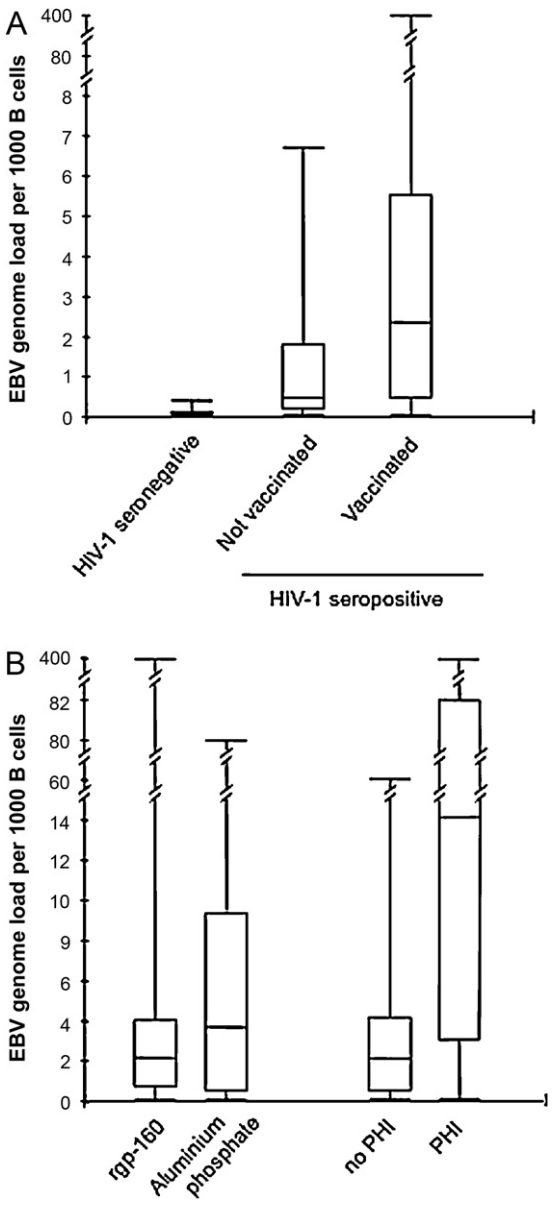

Fig. 1. Boxplot representing median EBV values and distribution of the data of the main patient groups compared in this study.
(A) HIV-l negative individuals, HIV-1 positive non- vaccinated individuals, and HIV-l positive vaccinated individuals. (B) Vaccinated individuals receiving recombinant gp160 vaccine, vaccinated individuals receiving "placebo" adjuvant alone (Alum), patients with documented symptoms upon HIV-1 primary infection (PHI), and patients who reported no PHI symptoms.

as compared to a median of 120×10^6 cells/L ($n = 18$) in the HIV-1 positive patients not included in the vaccine trial. We observed no significant correlation between the CD4+ cell counts and the EBV load, although there was a tendency to inverted correlation between these variables that patients with a high EBV load had low CD4+ cell counts, and patients with a low EBV load had a high CD4+ cell count.

The highest EBV values were exclusively found in the immunised group, while low values could be seen both in immunised and non-immunised patients. In the non-immunised HIV-1 carriers, the asymptomatic patients had a median EBV load of 0.19 copies/1000 B-cells ($n = 8$) while patients with AIDS-diagnosis had 0.98 copies/1000 B-cells ($n = 10$).

Notably, patients who received adjuvant alone "placebo" (i.e. alum) demonstrated an even higher EBV load (median 3.7 copies, $n = 16$) than those who received rgp160 (also with alum; median 2.1 copies, $n = 26$; Fig. 1B).

In general HIV-infected patients showed a higher EBV-DNA load in their B-lymphocytes than controls. In the control group the median EBV load was 0.049 per 1000 B cells ($n = 10$, Fig. 1A), while the median value for all the HIV-l infected patients was forty times higher, 2.0 per 1000 B cells ($n = 60$), a highly significant difference ($p < 0.0001$). Sex, age, origin of the individuals, and insufficient antiretroviral treatment did not affect the EBV load. One patient had a confirmed diagnosis of lymphoma at the time of blood sampling. This patient's EBV load was 53 copies per 1.000 B cells.

The inter-individual variation was large between HIV-1-patients, ranging over 10,000-fold (Fig. 1A), from 0.027 to 400 EBV copies per 1000 B cells. Forty percent (24/60) of the HIV-1 positive individuals had the same range of EBV load as the controls.

The difference in EBV load between symptomatic and asymptomatic groups of HIV-1 patients was relatively small, however a tendency to higher load in the asymptomatic group was noted [2.0 copies ($n = 45$) vs. 1.2 copies per 1000 B cells ($n = 15$), respectively]. The asymptomatic groups also showed a higher CD4 cell count. This paradoxical finding may be explained by vaccine effects, which will be discussed later. The data from all the patient subgroups are summarised in Table 3.

Immunised patients with a history of symptomatic primary HIV-infection (PHI) had a median value of 14 copies per 1000 B cells ($n = 8$), while the immunised individuals with no such history had a significantly lower median value of 2.1 copies per 1000 B cells ($n = 34$, $p < 0.05$; Fig. 1B).

For patients in the vaccine trials with an asymptomatic HIV-1 infection lasting for longer than ten years, EBV load was somewhat lower (median 1.5 copies, $n = 8$) in comparison to individuals with an asymptomatic infection lasting for a shorter period of time (median 2.4 copies; $n = 34$). No statistically significant differences were found.

Antibody titers to EBV-antigens were determined in all patients included in the vaccine trials, at the time of sampling for EBV-DNA-load. Nine patients had IgG anti-EA titers >1:80, ten anti-VCA titers >1:640 and three had elevated anti-p107 (EBNA 1)-titers in an ELISA-test. Although this did not correlate to EBV-DNA load, HIV-1 RNA levels or type of vaccine, the five patients with the highest levels of EBV DNA-load also had higher antibody titers.

Thirty-three patients were also tested for EBV-DNA in blood plasma. No EBV-DNA was detected in any of these samples. All of the saliva samples (throat washes) from HIV-1 infected patients were EBV-DNA PCR positive, but there was only a four-fold variation between the patients tested, and no co-variation with EBV DNA-load in B-lymphocytes (data not shown).

4. Discussion

Although HIV-1 infected patients seem to have significantly higher EBV load than controls, there is a stepwise increase from the time of HIV-1 infection to AIDS [19]. During the last decade the pathoimmunologic aspects on HIV-infection emphasise the B-cell involvement in addition to the T-cell deficiency. Polyclonal B-cell activation is a well-known consequence of HIV-infection, including hypergammaglobulinemia and increased production of autoantibodies [13,20]. Furthermore, the B-cell function in HIV-infected patients can be impaired as a result of exhaustion due to chronic persistent infection and apoptosis. Resting memory B-cells are particularly vulnerable in favour of activated B-cells, short lived plasmablasts and exhausted memory B-cells [13]. Immature, transitional positive B-cells undergo a development to CD21+ and later CD20 + CD19- B-cells [21], in analogy with PTLD in post-transplant patients [22]. As a result, the B-cells show a decreased ability to

Table 3
Averages of EBV DNA load in blood B-lymphocytes divided according to different patient groups.

	No of pat	EBV load per 1000 B cells
Immunised	42	2.4**
Non-immunised	18	0.49**
Immunised		
Asymptomatic	37	2.4
Symptomatic (AIDS)	5	2.0
Placebo	16	3.7
Vaccine	26	2.1
With PHI	8	14*
Without PHI	34	2.1*
Long term asymptomatic	8	1.5
<10 years asymptomatic	34	2.4
Non-immunised		
Asymptomatic	8	0.19
Symptomatic (AIDS)	10	0.98
HIV-1 seronegative controls	10	0.049***
HIV-1 seropositive	60	2.0***
HIV-1 seropositive		
Asymptomatic	45	2.0***
Symptomatic (AIDS)	15	1.2***

*** = $p < 0.001$
** = $p < 0.01$
* = $p < 0.05$

react to specific antigens, and this specific memory B-cell loss is not reversed by antiretroviral therapy [23].

Earlier publications suggest that vaccination by itself might lead to a similar polyclonal B-cell activation [24,25]. Thus, any vaccination might have a synergistic effect with the HIV-infection on the B-cell homeostasis. Alum, as a vaccine adjuvant, has also been linked to the development of cutaneous pseudolymphoma of B-cell origin probably via the induction of a Th2 response [26].

Vaccination of HIV-patients with tetanus or pneumococcal antigen as well as bacteriophage immunisation, have caused an increase of the HIV-1 RNA levels [27–29]. However, the effect of single as well as repeated vaccination on EBV load in healthy individuals is unknown. To the best of our knowledge, no general vaccination program exists where individuals are exposed to vaccine, and thereby alum, as frequently as in therapeutic HIV-1 vaccination trials, as in our study (4–6 administration/year).

The inter-individual variation between the patients in our study is considerable: the lowest quartile of EBV load in HIV-1 infected including AIDS-patients show similar values compared to the controls. It has previously been shown in homosexual male patients that the relationship between individual EBV load values ("set points") was maintained after HIV-1 seroconversion and also after initiation of antiretroviral treatment [30]. The EBV load in our study does not correlate well to the T-cell status of the patients, and therefore additional factors affecting the EBV load must be considered.

One such concomitant factor seems to be the therapeutic vaccination itself. In vaccinated patients there was a surprisingly similar influence of the vaccination in those who received only the adjuvant (alum) and those who got the adjuvant with the recombinant protein. This phenomenon has not been observed in non-vaccinated PHI patients. A history of PHI among the patients further significantly affected the EBV-host relationship, which was not observed in non-vaccinated PHI patients [31].

Although we followed several of the vaccinated patients for 2–3 years, we cannot make any conclusion concerning the persistent effect of immunisation on EBV DNA load. All analysed patients were introduced on cART soon after ending the vaccine trials. The introduction of cART affects the EBV host balance via the restoration of the CD4+ positive cells. This is most likely a strong confounding factor on the effect of immunisation on the EBV DNA load.

The immune stimulation caused by rgp160/alum may affect EBV in two ways. It may be either through influence on EBV replication resulting in infection of more B cells, or EBV infected B lymphocytes may be stimulated to proliferate through the activation of helper T-cells as a result of a Th2 enhancement by the vaccine. It has been shown that gp160 HIV-vaccination up-regulates immune activation T-cell markers, such as MHC class II and CD38 helper T-cells [32]. In an experimental prophylactic vaccination with gp120 in mice, the Th2-arm was activated [33]. The effects of therapeutic vaccination on T-cells might generate B-cell activation through non-specific immune stimulation in HIV infected individuals, as also shown for patients with autoimmune disease [15,32].

Our method detects B cell-associated EBV genome load. The method does not distinguish whether an expansion of EBV load in infected cells was caused by an increased copy-number or if it was caused by an increased number of infected cells. Using the same PCR method in a study of blood from healthy donors, we have shown that the number of EBV genome copies vary between 1–5 copies per B cell in different B-cell subsets [34]. It is not known if this is also valid in HIV-1 infected patients.

EBV-DNA PCR is a useful tool for monitoring clinical course of lymphoproliferative disease and for identifying patients at risk for tumours [11,35]. Measurement of EBV genome levels is then usually performed in extra-cellular plasma as cell free virus DNA [35,36]. However, Stevens et al. [11] concluded that serum may not be an optimal clinical specimen for EBV DNA load-monitoring because it does not consider the presence of cell-associated virus, and uncontrolled cell lysis may give irreproducible results or overestimation of the DNA load. However, we could not detect any EBV-DNA in plasma from our patients, which might reflect their relatively intact immune status. EBV DNA is rarely if ever detected in plasma from healthy individuals [37]. Cell-free virus DNA is probably only detected when released from dying cells in EBV carrying tumours or when the EBV host balance is significantly disturbed. Free virus may also be derived due to the replication of virus in sites outside blood in hosts with relaxed control of EBV-latency.

Monitoring of EBV load in B cells directly reflects viral load in the cell population, which most likely contains precursors of potential B-cell lymphomas. As mentioned above, HIV-1-patients do show both quantitative and qualitative variability of the B lymphocytes [13,38]. To circumvent this problem we analysed the load in CD19+ B cells. The chronic B-cell activation together with the loss of EBV immunoregulatory control seems to play a major role in the development of EBV-positive NHL in HIV-1 infected patients [39]. Excessive expansion of EBV-infected B-cells together with a risk for chromosome translocations conferring a malignant phenotype might explain the increased frequency of B-cell malignancies [8,40]. Our results must be considered in view of the well-documented decrease of lymphomas paralleled with the reconstitution of the immune system observed after the implementation of cART.

5. Conclusions

The major conclusion from our results is the recommendation to combine EBV-load analysis together with a long-term follow up of lymphoma risk in all therapeutic HIV-vaccine trials with or without combination anti-retroviral therapy.

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